FADD Is Required for DR4- and DR5-mediated Apoptosis

LACK OF TRAIL-INDUCED APOPTOSIS IN FADD-DEFICIENT MOUSE EMBRYONIC FIBROBLASTS*

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TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a member of the tumor necrosis factor family that can kill a wide variety of tumor cells but not normal cells. TRAIL-induced apoptosis in humans is mediated by its receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2). What constitutes the signaling molecules downstream of these receptors, however, remains highly controversial. Using the FADD dominant negative molecule, several groups have reached different conclusions with respect to the role of FADD in TRAIL-induced apoptosis. More recently, using FADD-deficient (-/-) mouse embryonic fibroblasts, Yeh et al. (Yeh, W.-C., Pompa, J. L., McCurrah, M. E., Shu, H.-B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W. S., Lowe, S. W., Goeddel, D. V., and Mak, T. W. (1998) Science 279, 1954–1958) concluded that DR4 utilizes a FADD-independent apoptotic pathway. The latter experiment, however, involved transient overexpression, which often leads to nonspecific aggregation of death domain-containing receptors. To address this issue in a more physiological setting, we stably transfected mouse DR4/5, human DR4, or human DR5 into FADD-/- mouse embryonic fibroblast cells. We showed that FADD-/- MEF cells stably transfected with TRAIL receptors are resistant to TRAIL-mediated cell death. In contrast, TRAIL receptors stably transfected into heterozygous FADD+/− cells or FADD−/− cells reconstituted with a FADD retroviral construct are sensitive to the TRAIL cytotoxic effect. We conclude that FADD is required for DR4- and DR5-mediated apoptosis.

TRAIL, also called Apo2L, is a member of the TNF family that can kill a variety of tumors but not normal cells, but its physiological function remains unknown. Several receptors that bind to TRAIL in humans have been identified. These include the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), and osteoprotegerin (2–4, 8–15). In mice, (data not shown) and others (16) have identified a gene encoding a protein with equivalent homology to human DR4 and DR5. Fas, TNF-R1, and DR3, the first three identified death domain-containing receptors, initiate apoptosis through recruitment of a common adapter protein FADD. FADD in turn recruits caspase 8 to form the death-inducing signaling complex, which leads to activation of a caspase cascade and eventual cell-death. FADD−/− MEFs are resistant to FasL or TNF-induced apoptosis and apoptosis mediated by DR3 overexpression. The role of FADD in TRAIL receptors, however, is highly controversial. The association between FADD and DR4 or DR5 has been inconsistent in the literature. Several initial papers showed that the dominant negative FADD protein failed to inhibit apoptosis initiated by DR4 and DR5 overexpression (2–4, 15). However, other groups reported inhibition of DR4/5 apoptosis in FADD dominant negative transfected cells (1, 5, 17). Use of the FADD dominant negative mutant raised concerns that it could potentially inhibit other proteins. Yeh et al. (7) overexpressed DR4 by transient transfection in FADD+/− MEFs, which led to apoptosis. The authors concluded that FADD was not required for DR4- and possibly DR5-mediated apoptosis. However, as transfection of a death receptor often leads to nonspecific aggregation of their death domains, it still is not clear if FADD is involved in the physiological setting of TRAIL-induced cell death. By utilizing FADD−/− and FADD+/− MEFs that are stably transfected with mouse DR4/5, human DR4, or human DR5, we show that FADD is essential for TRAIL-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Generation of Mouse Embryonic Fibroblasts—FADD+/− and FADD−/−

Day 9.5 embryos were genotyped by polymerase chain reaction (18) and mechanically disrupted by passage through a 26-gauge syringe in 1 ml of trypsin solution. The cells were cultured and passaged in complete Dulbecco’s modified Eagle’s medium with 10% FCS. The presence or absence of the FADD protein in these cells was confirmed by Western blot using anti-FADD antibodies.

Cloning—We used the human DR4 sequence to perform a BLAST search of the mouse EST data base to obtain a clone AI45286. Primers from the EST sequence were used to perform 5′ and 3′ rapid amplification of cDNA ends-PCR using a mouse spleen cDNA library to obtain a longer clone. This longer clone was used to screen a thymus cDNA phage library to obtain a full-length cDNA clone, which was cloned into pCI-HA. This HA-mDR4/5 was then cloned into the pNeo1 site of MSCV-puro-PLAP. BOSC cells were transfected with calcium phosphate with empty vector or MSCV-mDR4/5-puro-PLAP to generate viral supernatants. FADD+/− and FADD−/− MEFs were infected with respective viral supernatants. The cells were maintained in puromycin selection (10 μg/ml) and analyzed for PLAP by flow cytometry. pCMV1-flag-DR5 and pCDNA3-flag-DR4 were co-transfected with pTK-Hygro by LipofectAMINE™ Plus. Cells were placed into hygromycin selection at 50 μg/ml. The mouse cDNA of FADD was cloned into the XhoI site of MSCV-zeo. Viral supernatants were obtained and used to infect ligand; TNF, tumor necrosis factor; FADD, Fas-associated death domain protein; FCS, fetal calf serum; MEF, mouse embryonic fibroblasts; EST, expressed sequence tag; PLAP, placental alkaline phosphatase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; MSCV, murine stem cell virus.
FADD−/− MEF cells stably transfected with hDR4 or hDR5. Batch cultures were selected in the presence of 100 mg/ml zeocin.

Antibody and Western Blot—The extracellular domain of mDR4/5 was obtained by PCR with gene-specific primer pairs for cloning into pGEX-2T to create a glutathione S-transferase fusion protein. The fusion protein was purified with glutathione-agarose beads and used as antigen to raise polyclonal antibodies in rabbits. For Western blot, cell extracts were generated using 1% Nonidet P-40 lysis buffer (18). Blots were secondarily probed with horseradish peroxidase-conjugated anti-rabbit antibodies and were developed with enhanced chemiluminescence.

Cell Count and Flow Cytometry—Cells were trypsinized and washed with flow buffer (1× phosphate-buffered saline with 2% FCS). The cells were then blocked in 10% goat serum for 15 min on ice and stained using mouse anti-human placental alkaline phosphatase (DAKO, clone 8B6) followed by goat anti-mouse phycoerythrin-conjugated antibody.

Cytotoxic Assays—Cells were treated with increasing amounts of human recombinant TRAIL (Biomol) in the presence of 500 ng/ml cycloheximide for 24 h at 37 °C. Cells were analyzed for viability by either the crystal violet or the MTT assay. MTT was added to each sample to a final concentration of 1 mg/ml, and the cells were incubated for 3–4 h at 37 °C. The medium was removed, and isopropyl alcohol was added. Following color elution, absorbance at 595 nm was measured using an enzyme-linked immunosorbent assay reader.

Annexin V Assay—Cells were stained for 15 min in 100 μl of annexin V buffer (annexin V buffer 1× phosphate-buffered saline with 2% FCS). The cells were then blocked in 10% goat serum for 15 min on ice and stained using goat anti-human phycoerythrin-conjugated antibody.

RESEARCH

We established that FADD+/− and FADD−/− MEFs were not sensitive to TRAIL. Additon of soluble human TRAIL to the mouse fibroblast line, L929, led to apoptosis but did not cause substantial death of either FADD+/− or FADD−/− MEFs even in the presence of cycloheximide. This could be due to either a lack or low level expression of TRAIL receptors. We therefore stably transfected both FADD+/− and FADD−/− MEFs with the mouse TRAIL receptor expression plasmid. We identified a full-length mouse TRAIL receptor cDNA by screening a thymus cDNA library with a mouse EST probe homologous to human DR4 and DR5. Sequencing of this clone showed that it is identical to the reported mouse DR5 TRAIL receptor (16). However, because its predicted polypeptide is 39% identical and 56% homologous to both human DR4 and DR5 alike, it is not clear if this gene is the mouse ortholog of human DR4 or DR5. We will call this gene henceforth as mDR4/5. In transient transfection of 293T cells, expression of this gene led to extensive apoptosis and PARP cleavage (data not shown). We then stably transfected mDR4/5 into MEF cells and established several individual clones. Two empty vector-transfected controls and three mDR4/5-transfected clones were chosen for further analysis. To detect the TRAIL receptor protein expression, polyclonal antibodies were generated using a GST fusion protein. The mDR4/5-specific antisemur detects a protein with the predicted molecular mass of 46 kDa (Fig. 1). This species is present only in extracts of mDR4/5 transiently transfected cells but not in nontransfected cells or MEF cells. A similar band was detected in three mDR4/5 stably transfected FADD heterozygous MEF cells (mDR2-2, mDR2-17, mDR2-30) and in three FADD−/− stably transfected cells (mDR2-4, mDR2-10, mDR2-11, Fig. 1).

To assess the sensitivity of these transfected cells to TRAIL-mediated cell death, we subjected them to increasing amounts of human TRAIL protein in the presence of the protein inhibitor cycloheximide. Human TRAIL has been shown to induce apoptosis in both human and mouse cells alike (19, 20). As controls, the same cells are also treated with cycloheximide alone. As shown in Fig. 2A, apoptosis can clearly be seen in mDR4/5 stably transfected FADD−/− cells, whereas control vector alone-transfected cells are resistant to TRAIL-induced cell death. In FADD−/− MEF cells, however, transfection of mDR4/5 did not confer TRAIL sensitivity. All three stably transfected cells are equally resistant to the same extent to TRAIL-mediated apoptosis as the vector alone-transfected cells (Fig. 2A, right panel). Similar results were also obtained by measuring annexin V, which detects translocation of phosphatidylserine from the inner to the outer leaflet of the cellular membrane in early apoptotic cells (Fig. 2B).

To see if the requirement for FADD in TRAIL-mediated apoptosis also applies to the human receptors, we expressed human DR4 or human DR5 into FADD-deficient MEF cells by...

A. A. Kuang, G. Diehl, J. Zhang, and A. Winoto, unpublished data.
DISCUSSION

The TNF receptor superfamily consists of many members with diverse functions. A subset of this family includes Fas, TNF-R1, DR3, DR4, and DR5, which all contain a death domain in their respective cytoplasmic tails. The signal transduction pathway of Fas-mediated apoptosis has been studied extensively (21–25). Fas forms a trimeric complex and upon stimulation by FasL, recruits FADD (26, 27), an adapter molecule consisting of a death domain and a death effector domain (28). FADD in turn interacts with a death effector domain-containing caspase (caspase 8), which activates a caspase cascade, leading to apoptosis (29, 30).

Although the role of FADD in Fas, TNF-R1, and DR3 has been clearly established (7, 31, 32), its role in TRAIL-mediated apoptosis has remained controversial. Whether FADD can interact with DR4 or DR5 is not clear, as different groups have reported conflicting data regarding the ability of FADD to associate with DR4 and DR5. Interaction by co-immunoprecipitation, however, can be difficult to detect because of the low abundance of the molecules or unfavorable lysis conditions. A case in point is the inability to detect the presence of FADD in the endogenous TNF-R1 complex (33, 34). Despite this, FADD−/− MEFs are resistant to TNF-induced apoptosis (7), suggesting that TNF receptor utilizes FADD indirectly in initiating apoptosis. Thus, genetic studies are more definitive in establishing the importance of a protein in a signal transduction pathway. Dominant negative FADD has been reported to inhibit TRAIL-induced apoptosis (1, 5, 6, 17). However, overexpression of DR4 in FADD−/− MEFs can still lead to cell death (7). To address this issue, we used FADD−/− MEFs in a stable transfection assay to establish the role of FADD in TRAIL-induced cell death. FADD−/− MEFs transfected with mouse DR4/5 are sensitive to TRAIL-mediated apoptosis in a dose-dependent manner. In contrast, FADD−/− MEFs expressing the mouse TRAIL receptor remain resistant to FADD. To correlate with the human system, we also transfected human DR4 or DR5 into FADD−/− cells. Despite expression of the TRAIL receptors, these cells remain resistant to TRAIL-mediated cell death. However, when these cells were reconstituted with the FADD molecule, they regained their TRAIL sensitivity. Thus, FADD is essential for the ability of TRAIL to initiate apoptosis. The discrepancy with the previous studies (7) is most likely due to the non-physiologically high level expression of the transiently transfected molecule, leading to either nonspecific tox-
icity or induction of an alternate pathway of apoptosis. Indeed, all receptors containing a death domain can induce apoptosis when transiently transfected into mammalian cells. However, in stably transfected cells or in physiological settings, expression of the respective receptors alone does not lead to cell death. Expression of the respective ligands in the same cells or contacting cells is necessary to initiate apoptosis.

While this work was in preparation, Bodmer et al. (35) reported that FADD and caspase 8 are recruited to the DR5 death-inducing signaling complex in Jurkat T cells that express DR5 but not DR4. Using these DR5-expressing Jurkat T cells deficient for FADD or caspase 8, they showed that these cells are resistant to TRAIL-mediated apoptosis. Their results confirm our findings with regard to DR5-mediated apoptosis. We extend these findings along with our FADD reconstitution studies to show that FADD is also required for DR4-mediated apoptosis.

In summary, we demonstrate that DR4- and DR5-mediated apoptosis depends critically upon FADD, and similar signaling pathways are used in both mouse and human cells. The essential role of FADD in apoptosis initiated by all the known death domain-containing TNF receptor family members may underscore the dramatic phenotype of FADD-deficient mice. These mice die by day 9.5 of gestation due to abnormal cardiac development (7, 18). In addition, T-cell receptor-mediated cell proliferation is defective in FADD-deficient T lymphocytes (18).

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